THE EFFICACY OF A LACTIC ACID BACTERIA TREATMENT TO INHIBIT *Escherichia albertii* ON GROUND CHICKEN

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Efficacy of a Lactic Acid Bacteria Treatment to Inhibit *Escherichia albertii* on Ground Chicken. (May 2014)

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Escherichia albertii has recently become a challenge to food safety. The organism has been mistakenly identified as *Hafnia alvei*, a member of the pathogenic *Escherichia coli* and others. Early last year, a foodborne disease outbreak in Japan identified *E. albertii* as the causative agent. Lactic Acid Bacteria (LAB) can be used as a natural antimicrobial applied to foods to inhibit the growth of pathogenic microorganisms. To date, no significant research has been performed as to whether the LAB have any effect on foods inoculated with *E. albertii*. Therefore, the objective of this experiment was to determine the efficacy of treatment of *E. albertii* with LactiGuard™, a natural LAB treatment currently being employed in the food processing industry to increase food safety. If successful, this will indicate that LactiGuard™ is an intervention treatment that can be used to control the growth of *E. albertii* on cross-contaminated poultry.

DEDICATION

I would like to dedicate this paper to my father who instilled within me the belief that there is nothing more incredible than the desire of a person to learn and to admire the world around them. He taught me to appreciate the little things in life and how to apply my schooling to the world around me. I would have never been able to accomplish this project without his guidance.

I would also like to dedicate this paper to my mother because no one but her could give me the undying love and support I have needed throughout my life. Simply put without her I would be lost.

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I would like to extend my thanks to Dr. Christine Alvarado and Mr. Gerardo Casco, Texas A&M University Department of Poultry Science, for their guidance regarding my procedure of chicken inoculation and for loaning me a small meat grinder and Peracetic acid concentrate.

NOMENCLATURE

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection, Manassas, Virginia
CFU	Colony Forming Unit
E. albertii	*Escherichia albertii*
E. coli	*Escherichia coli*
H. alvei	*Hafnia alvei*
LAB	Lactic Acid Bacteria
lbs	Pounds
$\log_{10}$	Logarithm (Base 10)
MRS	deMan, Rogosa, and Sharpe Lactobacilli agar
RR-MAC	MacConkey agar supplemented with 0.1 grams/liter Rifampicin and 10 grams/liter L-Rhamnose
TSA-R	Tryptic Soy Agar supplemented with 0.1 grams/liter of Rifampicin

CHAPTER I

INTRODUCTION

In 1990, a diarrheal sample containing *Escherichia albertii* was obtained from a nine month old girl and isolated at the International Center for Diarrhoeal Disease Research (ICCDR) in Bangladesh. The pathogen contained within the stool samples was originally identified as *Hafnia alvei* due to presumptive identification along with *H. alvei* being the only organism isolated from the child *(7)*. Further research by Albert and others *(8)* led to the discovery that the organism produced attaching and effacing (A/E) lesions that were genetically similar to those produced by enteropathogenic *Escherichia coli* (EPEC). This discovery more closely aligned the organism, thought to be *H. alvei*, with the enteropathogenic *E. coli*. Genetic testing showed that *E. albertii* possessed enough genetic differentiation in its genome, compared to E. coli, to be named as a new species under the genus *Escherichia*, resulting in the suggestion of *Escherichia albertii*, named for M.J. Albert, the leader of the group originally isolating and identifying the pathogen *(4)*. The naming of a new species naturally attracted many researchers to further identify the organism. Abbott and others *(1)* found that *E. albertii* was not able to ferment D-sorbitol, a trait that is typically closely associated with some strains of *E. coli*. It was later found, through genetic typing of the 16s RNA sequence, that *E. albertii* is most closely related to *Shigella boydii* not *E. coli (6)*.

Recently, *E. albertii* was identified as the causative agent of an outbreak of foodborne disease in Japan where 31 people became ill with diarrhea, fever and abdominal pain *(11)*. Although it is not known what food acted as the transmission vehicle for the outbreak, the outbreak has been

traced back to a restaurant in Japan where it has been theorized that the food became contaminated at some point in the preparation process. From this outbreak, Ooka and others *(11)* recovered 26 *E. albertii* isolates, 13 from symptomatic humans, that had been previously misidentified as EPEC. Previous international research by Ooka and others *(10)* found that the cause of around 9.4% of foodborne illnesses may be misdiagnosed in the laboratory setting because of the similarity of *E. albertii* to other more well-known gastrointestinal pathogens such as *E. coli* O157:H7. Although it is not known what caused the outbreak in Japan, there is much concern regarding whether avian species are a potential vector of the infectious agent *(9)*. Young and others *(9)* found that wild birds can carry E. albertii and can be a potential source of infections in humans. Clearly, *E. albertii* is an under-researched pathogenic microbe whose prevalence is mostly unknown due to its lack of epidemiological data and misidentification. This has contributed significantly to a lack of research on food intervention treatments targeting *E. albertii*.

The Centers for Disease and Prevention (CDC; Atlanta, GA) estimates that 9.4 million cases of foodborne illness occur in the United States every year from major pathogens, where roughly 1,351 of these cases result in death of the consumer and 64% of these deaths are caused by bacteria alone *(14)*. Estimates of all foodborne illnesses in the United States annually approach 48 million cases *(14)*. Scharff and others *(15)* used these same estimates to conclude that foodborne illnesses account for $77.7 billion in annual health-related costs. It is worth noting that of all food commodities produced in the United States, poultry products account for the majority (19%) of all food related deaths *(12)*. This leads to a problem when considering today's health mindset where consumers desire a healthier food product, usually poultry, treated with

more natural antimicrobials. Common poultry treatments in today's processing plants includes varying chemical washes or sprays such as acidified sodium chlorite or cetylpyridinium chloride. Natural preservative include the use of plant based preservatives, garlic and bacteria.

The Lactic Acid Bacteria (LAB) have been known to compete for attachment sites and resources with other microbes and, in some cases, their waste products hinder or inactivate certain bacteria *(3)*. LAB were first studied due the inhibitory effect of nisin, a bacteriocin produced by Lactobacillus lactis *(2)*. This bacteriocin is ideal for use because it created by a nonpathogenic organism and is easily digested by humans. Lactic acid and other products produced by LAB have been used to lower the pH and have been found to be effective *(5)*. To date, no research has been conducted with the aim of testing whether LAB will have any effect on *E. albertii* in any food product. The purpose of this experiment was to determine LactiGuard™ would produce any statistically significant change in *E. albertii* cell counts inoculated onto ground chicken products during refrigerated storage. LactiGuard™ is a natural antimicrobial consisting of four non-pathogenic microbes, which are known to antagonize foodborne pathogens: *Lactobacillus acidophilus* (LA51), *Lactobacillus lactis* (NP7), *Pediococcus acidilactici* (D3) and *Lactobacillus animalis* (NP28). The efficacy of spraying LAB on *E. albertii* can determine the usefulness of using LAB as a step in food processing to reduce prevalence of *E. albertii*.

The research hypotheses to be tested are H_0: the application of LAB will not show a statistically significant reduction of *E. albertii* on inoculated ground chicken, and; H_1: application of LAB onto *E. albertii* inoculated chicken will produce a significant reduction in the ability of *E. albertii* to reproduce. The hypotheses will be confirmed or rejected based on Analysis of

Variance (ANOVA) of experimental results from experiments enumerating surviving *E. albertii* on ground chicken meat inoculated with the pathogen and treated with the LAB antimicrobial ($p<0.05$).

CHAPTER II

METHODS

Preliminary trials methodology

Growth curve

Three strains of rifampicin resistant *E. albertii* (ATCC 19982, 9194 and 10457), obtained from the Department of Animal Science Food Microbiology Laboratory, were resuscitated from cryogenic beads by making two successive transfers into 9.0 mL TSB (Becton, Dickinson and Company., Spark, Maryland) for incubation at 35°C for 24 hours. After revival, the *E. albertii* strains were streaked on TSA (Becton, Dickinson and Company., Spark, Maryland) plus 0.1g/L rifampicin (Sigma Aldrich., St. Louis, Missouri) to ensure antibiotic resistance. Colonies from each plate/strain were aseptically transferred to slants of TSA with an overlay of mineral oil for storage in the refrigerator (5°C).

Two days before the trial was initiated the E. albertii cells were revived via two successive subcultures in TSB for 24 hours each at 35°C. The cells were then washed twice by adding 9 mL of 0.9 % NaCl (Becton, Dickinson and Company., Spark, Maryland) following centrifugation at 2209 RCF for 15 minutes. The cell cultures were serially diluted (10 fold) six times where the final dilution was into 99 mL of TSB (100 fold). Samples were then taken at 0, 1, 2, 4, 6, 8, 10, 12 and 24 hours for dilution and plating on TSA. The 0 and 24 hour time points were also plated on RR-MAC.

Achieving target application of LactiGuard™

The first preliminary trial was run to determine the concentration of inoculum and sprays needed to reach 10^6 CFU/g of LactiGuard™ on chicken. The four isolates of Lactic Acid Bacteria (LAB): *Lactobacillus lactis*, *Lactobacillus animalis*, *Lactobacillus acidophilus* and *Pediococcus acidilactici*, were revived by aseptically adding 1 gram of each isolate to individual 99 mL bottles of 0.1% (w/v) peptone (Becton, Dickinson and Company., Spark, Maryland). After shaking vigorously each bottle, 10 mL of each isolate were combined together in a 50 mL conical tube. One milliliter of this cocktail solution was added to 9 mL of 0.9% (w/v) NaCl. One milliliter of this diluted solution was then added into 99 mL of NaCl which was subsequently added into a plastic spray bottle, obtained from a College Station, TX, retail grocer, for application.

Raw chicken trimmings were obtained from the Department of Poultry Science, Texas A&M University, College Station, TX, for this experiment. Upon arrival to the Food Microbiology Laboratory, chicken trimmings were stored at -18°C until needed. Two days before use, the chicken trim was set in the 5°C cold room to thaw. After 24 hours, the chicken trim was taken out of the cold room and aseptically weighed into four distinct samples each totaling 250 grams, after which the chicken aliquots were placed back in the cold room. One sample served as the negative control, to determine the background LAB present in the chicken. The other three samples were sprayed (1.6 mL/spray) with LactiGuard™ (roughly 9.2 $\log_{10}$ CFU/mL) either one, two or three times followed by homogenization of the treatment by hand massaging for 1.0 minutes. After massaging, three 25.0 g aliquots of each sample were removed for serial dilution (10 fold) in 0.9% NaCl and were plated on MRS (Becton Dickinson and Company., Sparks,

Maryland). The trial was replicated three times (n=3) and plate counts of organisms were converted into $\log_{10}$ for statistical analysis.

Inoculation of chicken trimming with E. albertii

Two days before the experiment was initiated the *E. albertii* strains were revived from the slants by two successive sub-cultures, the first of which occurred in 9 mL TSB. The second transfer was completed in a 50 mL conical tube containing 45 mL of TSB with phosphate added to the amount of 4 g/L total phosphate in solution. The phosphate has been added to ensure that the bacteria do not become acid resistant during incubation, from acid waste buildup, due to the acid fermentation of glucose present in the medium. After incubation for 24 hours, the conical tubes will be centrifuged for 15 minutes at 2209 RCF. The supernatant will be poured off so the cells can be re-suspended in 45 mL of 0.9% NaCl. After re-suspension the third time, 15 mL from each tube will be transferred into two sterile 50 mL conical tube. These 45 mL cocktails will be centrifuged and re-suspended following the same procedure as above. Two distinct bottles of inoculum solution were prepared. The first bottle was prepared by adding all 45 mL of cocktail into 405 mL NaCl creating a 10^8 CFU/ml solution. The second bottle was made by adding 4.5 mL of cocktailed solution into 445.5 mL of NaCl creating a 10^7 CFU/mL solution. Chicken trimming was obtained, thawed and weighed in the same method as stated previously where only three 300 gram aliquots were prepared. One sample acted as the negative control and was used to determine if background organisms resistant to rifampicin were present. Chicken inoculation was achieved by pouring each bottle into their respective bags for submersion totaling 1.0 minutes. The chicken was allowed to dry for one minute. Two 25 gram aliquots were taken and serially diluted followed by plating on TSA-R. The remaining 250 grams of each sample were

ground with a 10 mm plate producing a coarse ground chub, after which two additional 25 gram aliquots were diluted and plated. The negative control was sampled before and after grinding following the same method. The trial was replicated three times (n=3) and plate counts of organisms were converted into $\log_{10}$ for statistical analysis.

Experimental methodology

Preparation of inoculum solution

The preparation of the inoculum solution followed the same protocol as the preliminary trial concerned with *E. albertii* application. Changes to be made are that only one 45 mL cocktailed solution is to be made and 0.9 ml of the re-suspended cocktail will be added to four bottles containing 899.1 ml of 0.9% NaCl totaling 900 mL of inoculating solution per bottle.

Preparation of treatments

The four isolates of Lactic Acid Bacteria (LAB) were revived by aseptically adding 1 gram of each isolate to individual 99 mL bottles of 0.1% peptone. After mixing each bottle, 10 mL of each isolate were mixed together in a 50 mL conical tube. Ten milliliters of this cocktail solution was added to 90 mL of NaCl which was subsequently added into a plastic spray bottle, obtained from a College Station, TX, retail grocer, for application. The change in dilution from the LAB preliminary trial was due to problems encountered concerning differences in sample weight and loss of cells during the increased drying times.

The peracetic acid treatment was prepared by adding 15 mL peracetic acid concentrate to 485 mL distilled water for an application concentration of 700 ppm. The solution was then placed in

a similar spray bottle for application. The peracetic acid was applied according to manufacturer's guidelines. The verification of concentration was achieved using the test kit provided by FMC. Ten milliliters of the solution to be tested were placed in a tube where 10 drops of sulfuric acid and one drop potassium iodide were added. Peracetic acid titrant was added on a dropwise basis with swirling of the solution following each added drop. The drops were added until a change in the solution's color was observed. The number of drops added was multiplied by 15 yielding the concentration in the original solution.

Preparation of chicken

Raw Chicken trimming was obtained from the same source as the preliminary trials. The same storage and aliquoting procedures from the preliminary trials were used. The first sample weighed served as the negative control and weighed 350 grams. The negative control was not inoculated with pathogen or treated with a food intervention agent and was used to determine the background LAB and possible antibiotic resistant organisms. The second sample will be treated with *E. albertii* inoculum and will weigh roughly 1050 grams. The separated samples were then placed back in the cold room for use the next day. The next day, the chicken from sample two was submerged in a 3.6 liter volume of *E. albertii* at a concentration of 10^6 CFU/mL for 1.0 minutes. The inoculated trimming was then allowed to set for 30 minutes (also allowing for bacterial attachment to meat surfaces) and then separated to dry into three 350 gram portions. One of the inoculated chicken portions was sprayed with Lactiguard™ to a target of 10^6 CFU/g and allowed to dry for 30 minutes. A second 350 gram portion of the inoculated chicken will be sprayed with peracetic acid and allowed to dry for 30 minutes. All the chicken samples were then ground in a grinder, with a plate size of 10 mm producing a coarse ground chub, and the

newly ground chicken was placed in the cold room. The ground chicken from each sample was then separated into ten 30 gram portions. Each portion was wrapped with saran wrap to model market conditions. Two 30 gram samples from each treatment were removed and subjected to serial dilution for plating on days 0, 1, 3, 5 and 7. The negative control was plated on MRS to determine the amount of background LAB. The positive control was plated on TSA-R along with the peracetic acid treated sample. The Lactiguard™ treated samples were plated on both TSA-R and MRS*. The MRS* was used for selective plating of LactiGuard constituent organisms and was supplemented with a series of antimicrobials protected under non-disclosure agreement with the intervention supplier (Nutrition Physiology Corp., Overland Park, KS).

Statistical analysis and experimental replication and design

The experiment was replicated two times (n=2) and plate counts of organisms were converted into $\log_{10}$ for statistical analysis. Data were assumed to adhere to a normal distribution and significantly different means were identified by one-way analysis of variance (ANOVA), with means being separated via Tukey's Honestly Significant Differences (HSD) test at $p=0.05$. All statistical analysis was completed using JMP v.11.1.1 (SAS Institute, Inc., Cary, NC).

CHAPTER III

RESULTS

Preliminary results

Determining growth after 24 hours

The results in Figure 1 show that the E. albertii grew roughly 6.5 $\log_{10}$ CFU/mL over 24 hours of incubation at 35°C. This data was used in later experiments to be able to approximate the amount of cells that are present in solution after incubation. The lack of uniformity at later sampling time may be attributed to sampling and plating error.

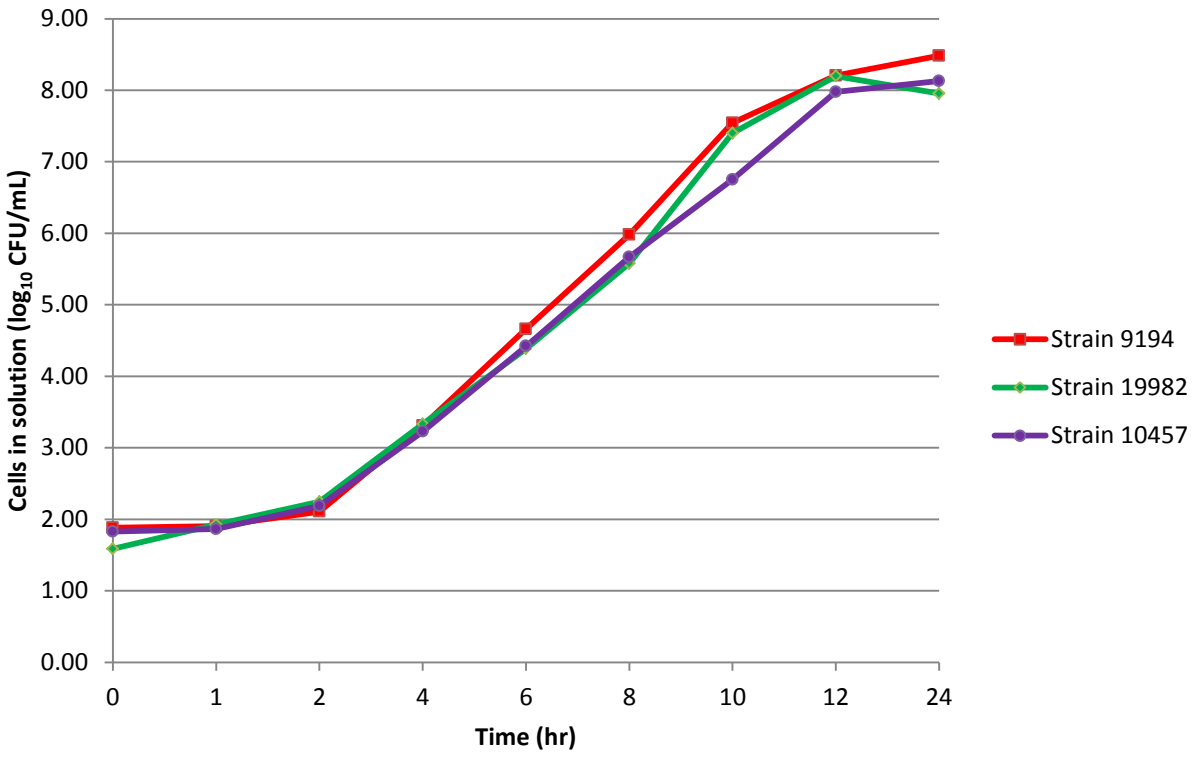

Figure 1. Growth curve of three *E. albertii* strains over 24 hours. A growth curve with triplicate means of three *E. albertii* strains is shown with data being taken at hours 0, 1, 2, 4, 6, 8, 10, 12 and 24.

Application of LactiGuard™

The negative control was plated on MRS to determine background LAB. Colony counts were within the acceptable range for normal background LAB present in poultry samples (Data not shown). The LactiGuard inoculum was found to total to roughly 9.2 $\log_{10}$ CFU/mL (Data not shown). The results of the treated samples indicate that three sprays of LactiGuard™ inoculum most closely approached the 6.0 $\log_{10}$ CFU/g application limit set by the USDA-FSIS (Figure 2). In the final trial, an addition chicken sample was sprayed four times but subsequent attachment numbers did not differ significantly from three sprays (Data not shown).

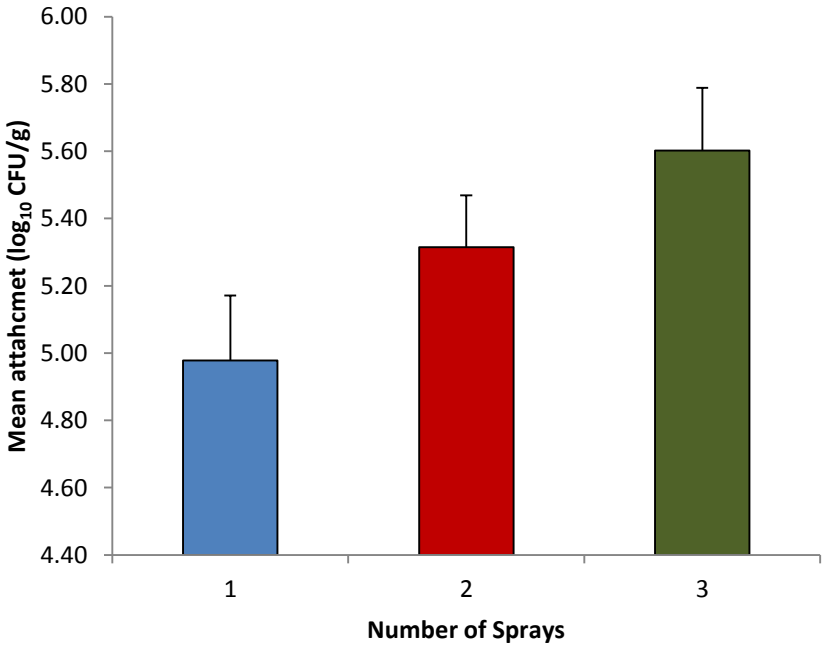

Figure 2. Mean attachment of LAB on poultry as a function of number of sprays. Bars depict mean attachment from triplicate identical replicates (n=3). Error indicate one SD from the mean.

Inoculation of poultry trimming with E. albertii

The negative control in this experiment was plated on TSA-R to determine background resistance to rifampicin. No resistance was found pre or post grind in all three replicates (Data not shown). Figure 3 depicts the attachment numbers of E. albertii pre and post grinding when applied at inoculum concentrations of either 10^8 or 10^7 CFU/mL. The data from this trial was used to create the inoculum solution for the experiment by further diluting the experimental inoculum to 10^6 CFU/mL. This would result in roughly 4.0 $\log_{10}$ CFU/g E. albertii attachment on the experimental chicken.

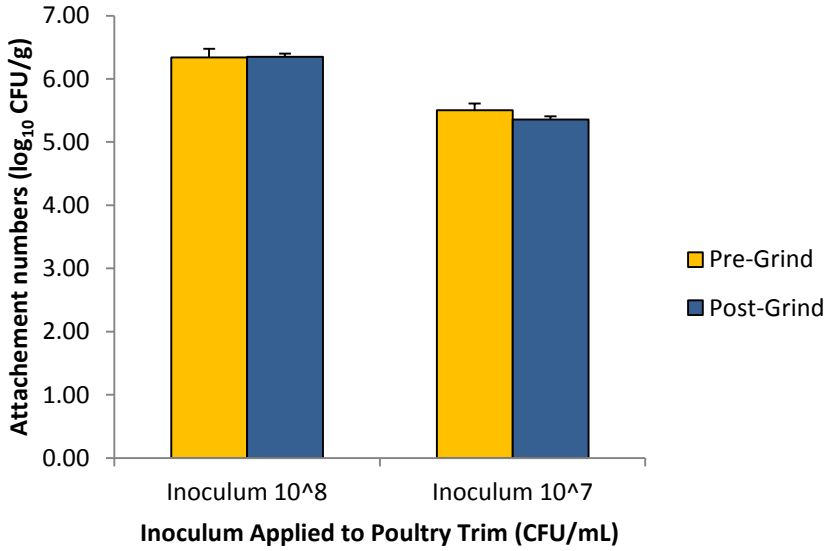

Figure 3. The attachment numbers of E. albertii applied at varying concentrations before and after grinding. Bars on the left depict E. albertii attachment number pre grind while bars on right depict attachment numbers post grind of triplicate identical replicates (n=3). Error bars depict one SD from the mean.

Experimental results

The negative control was plated on both TSA-R and MRS* to determine background antibiotic resistance. No background microbes bearing resistance to rifampicin were recovered on TSA-R surfaces. Conversely, multiple colonies on MRS* from non-treated meat samples were recovered on MRS*, indicating background LAB capable of resisting the antibiotics, thus indicating lack of strong selective capacity of MRS* for selective recovery of LactiGuard™ (Data not shown). The results of the treated samples indicate that neither LactiGuard™ or peracetic acid treatment produced any significant inhibitory effect on *E. albertii*, or its survival on poultry meat (Figure 4). Statistical analysis of gathered data indicated that duration of sample incubation was not significant in its effect on *E. albertii* survival or inhibition ($p \geq 0.05$) (Data not shown). Although the LactiGuard™-treated samples had a higher number of *E. albertii* survivors, this would be attributable to inherent sampling and plating variability and/or lack of reproducibility. Over seven days, the numbers of *E. albertii* cells did decrease but compared to the control these decreases were not a result of treatment. Perez and others *(13)* found that *E. albertii*, when placed under refrigeration conditions, will slowly die over time. Smith and others *(16)* found that, under similar treatment and storage conditions, *E. coli* and *Salmonella* were reduced by 2.0-3.0 $\log_{10}$-cycles in a beef matrix. It is possible that the chicken could have caused reduced LactiGuard™ efficacy due to differences in nutritional make-up of the chicken matrix as compared to beef. Additionally, Smith and others *(16)* were able to treat their beef samples to 10^8 CFU/g due to differences in USDA-FSIS allowances between chicken and beef. Further, given the inoculation of trimmings and treatment with LactiGuard™ prior to grinding, the opportunity for dispersion of cells through grinding may have led to insufficient numbers of LAB contacting pathogens for inhibition to be observed.

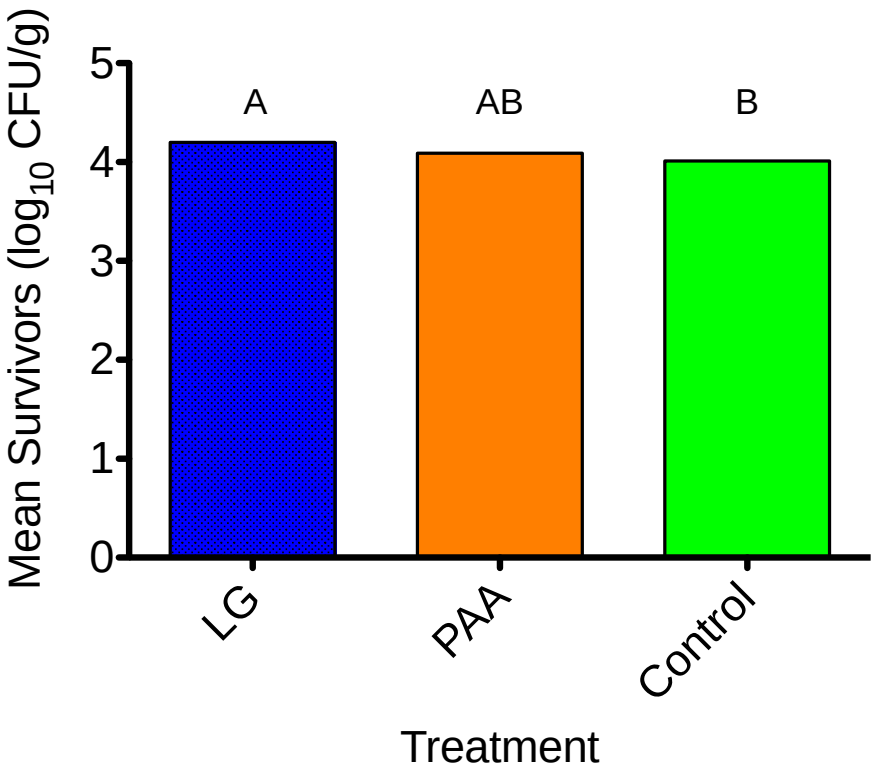

Figure 4. Least square means of *E. albertii* survival on poultry meat as a function of antimicrobial treatment. Bars depict LS means from duplicate identical replicates (n=2). LG = LactiGuard™ 6.0± 0.1 $\log_{10}$ CFU/g inoculum; PAA = peracetic acid (700 ppm). Bars not sharing letters differ at $p<0.05$.

Figure 5 shows the growth of LactiGuard™ on poultry inoculated with *E. albertii* over the seven day storage period. The LactiGuard™ was applied at 10^6 CFU/g on day zero, but over seven days the cells increased to 10^7 CFU/g. This growth can be attributed to the psychrophilic qualities of the cocktailed isolates. This growth can have severe consequences in an industry setting due to USDA-FSIS application guidelines stating that LAB treatments can only be applied on poultry to a total of 10^6 CFU/g. A future experiment of the efficacy of LactiGuard™ on *E. albertii* would benefit from being carried out on a beef food matrix so that results from Smith et al. will be more useful.

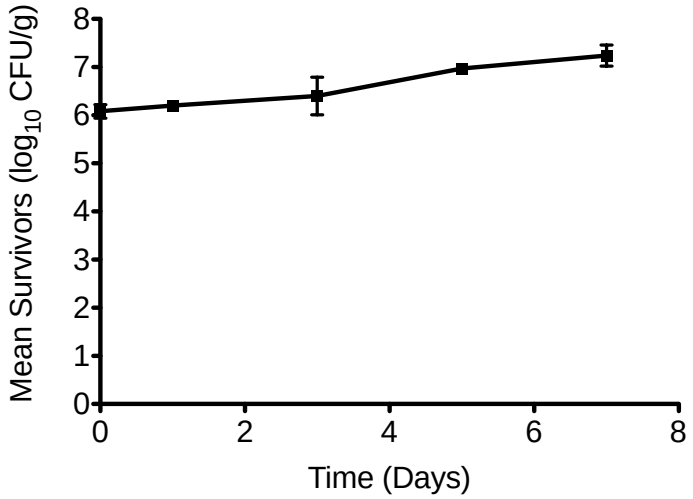

Figure 5. Inoculation and increase in numbers of LactiGuard™ on *E. albertii*-inoculated poultry meat at 5°C. Symbols indicate means of duplicate identical replications (n=2); errors bars indicate one sample standard deviation. LactiGuard™ were enumerated on MRS agar supplemented with proprietary antibiotic mixture.

CHAPTER IV

CONCLUSIONS

The purpose of this study was to determine the efficacy of a LAB treatment to inhibit *E. albertii* inoculated chicken. *E. albertii* is a foodborne pathogen that is resistant to some common biological and chemical preservative methods employed by the poultry industry. Hopefully, future studies will be able to provide insight into why *E. albertii* is more resistant to the fermentative byproducts of LAB. This may provide a novel testing method to allow for easier differentiation between *E. albertii* and the closely related *E. coli*. LactiGuard™ as a food intervention step to inhibit *E. albertii* may have better efficacy when applied on carcasses or in a post grind setting.

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